Neonatal mycobacterial specific cytotoxic T-lymphocyte and cytokine profiles in response to distinct BCG vaccination strategies

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SUMMARY

This study evaluated whether different bacillus Calmette–Guérin (BCG) strains, routes of administration, vaccination age and percutaneous tools influenced immune responses to BCG vaccination in infants. Proliferative responses, cytokine production and cell-mediated cytotoxicity obtained in post-vaccinated children were compared to baseline cord bloods and unvaccinated 10-week-old infants. BCG vaccination generally induced strong lymphoproliferative and T helper type 1 (Th1)-type cytokine responses. There was a trend for greater responsiveness following the intradermal route of vaccination, with Japanese-172 strain and with delaying vaccination until 10 weeks. Cord mononuclear cells differentially stimulated the Th2-type cytokines interleukin-5 (IL-5) and IL-10 selectively in response to BCG, as compared to H37Rv or purified protein derivative stimulation. We document for the first time the generation of mycobacterium-specific cytotoxic T lymphocytes in neonates, following BCG vaccination. Cytotoxic activity correlated with the ratio of interferon-γ to IL-5, aside from a single instance where use of the Biovac® tool resulted in a striking dissociation selectively against H37Rv targets. These data have implications for correlates of protective immunity in design of vaccine studies.

INTRODUCTION

Developing countries have witnessed an escalation of the tuberculosis epidemic, particularly in sub-Saharan Africa.[1] Tuberculosis has also re-emerged in industrialized countries due to a number of factors, including the spread of human immunodeficiency virus (HIV).[2] This has led to renewed interest in vaccines aimed at preventing tuberculosis. While current Bacillus Calmette–Guérin (BCG) vaccines prevent the invasive complications of childhood tuberculosis, such as meningitis and miliary disease, they only provide variable protection against adult pulmonary disease.[3,4] Variable BCG vaccine efficacy has been attributed to variation in BCG vaccine strain, host genetic factors, genetic variation in strains of *Mycobacterium tuberculosis*, or a masking effect due to environmental mycobacteria.[4]

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While research efforts are currently directed at new vaccine candidates,[5] it may be possible to improve the immunogenicity of the existing BCG vaccine. Factors such as vaccine dose, age at vaccination, and route of administration may also impact on the immune response, but these have not been systematically studied.

T helper type 1 (Th1)-type responses, characterized by the production of interferon γ (IFN-γ), have been considered to be required for protection against mycobacterial disease.[6] Obstacles to successful neonatal vaccination include immaturity of T-cell immunity resulting in difficulty in priming neonatal T cells and bias of neonatal immunity towards a Th2-type response.[7–9] Immunological responses are known to be governed by factors such as the dose and route of vaccine administration. Low-dose BCG vaccination in experimental animal models has been associated with a Th1-type response while higher doses have been associated with a mixed Th1- and Th2-type response.[10] In adults, high doses of percutaneous BCG were required for an appropriate Th1 response.[11] There is some evidence to suggest that intradermal BCG vaccination, the route recommended by the World Health Organization, may be a more efficient method of vaccine delivery (as measured by

tuberculin skin tests) than percutaneous BCG vaccination.[12] A recent study demonstrated for the first time Th1-type responses in human neonates receiving BCG vaccination at birth or at 2 months of age.[13] However, knowledge of the correlates of protective immunity against the development of human tuberculosis is still incomplete and is the subject of ongoing discussion and research.[14] Th1-type responses (exemplified by T-cell proliferation and IFN-γ production) may be necessary but not sufficient for protective immunity. Cytotoxic T lymphocytes (CTL) that recognize and lyse infected macrophages have also been considered important in protective immunity against tuberculosis.[15,16] CTL have been isolated from patients with active tuberculosis.[17,18] There are reports of CTL induction in humans following BCG vaccination, but to date these have only involved adult subjects.[19,20]

The aims of this study were therefore threefold. First, to determine whether cellular immune responses vary according to BCG strain (Japanese 172 or Danish 1331), route of administration [percutaneous (p.c.) or intradermal (i.d.)], type of tool used for p.c. vaccination (Bignell® or Biovac®), or age at vaccination (birth or 10 weeks) in a group of infants. Second, to ascertain the balance of Th1-type and Th2-type cytokines under these distinct vaccination conditions and third, to ascertain if BCG vaccination primes for CTL response in human neonates.

MATERIALS AND METHODS

Study design

Infants/subjects. This study was conducted at a community-based, primary-care service. Healthy new-borns were recruited. Exclusion criteria for enrolment were birth weight below 2500 g, a family history of tuberculosis or known HIV infection. Five groups of children were immunized: 11 new-borns were given the Danish BCG strain i.d. at birth (DID); 11 new-borns were given the Danish strain i.d. at 10 weeks of age (del DID); 10 new-borns were given the Japanese BCG strain i.d. at birth (JID); 10 new-borns were given the Japanese strain by p.c. at birth using the Bignell® tool (JPC Big); 20 new-borns were given the Japanese strain by p.c. vaccination at birth using the Biovac® tool (JPC Bio). The Bignell® tool, manufactured in the UK, is a spring-loaded, multi-puncture device with 18 steel needles. The Biovac® tool is a locally manufactured multi-puncture device with nine needles and its skin penetration is determined by manual pressure. Peripheral blood (10 ml) was taken from all infants 10 weeks after BCG immunization and also prior to BCG immunization in the group immunized at 10 weeks (unv 10 week). After delivery of the infants (except for the JPC Bio group) 20 ml of cord blood was collected from the umbilical vein of the placenta. As there was no difference between the groups, all the results of the cord bloods were pooled (cords) in all instances except where the effect of maturation was to be investigated using cytokine responses from the same group of 10 infants (del DID) examined at three time points. These were cord blood, pre-BCG immunization (unv 10 week),

and 20 weeks of age (10 weeks post BCG immunization, del DID). Written informed consent was obtained from the mothers of the infants enrolled into the study. The study was approved by the Ethics and Research Committee, University of Cape Town and their guidelines for clinical research were adhered to.

Vaccines and immunization tools. BCG vaccine (0·05 ml) Danish strain 1331 or Japanese 172 was injected intradermally via a 26-gauge needle into the lower deltoid region of the right arm at the insertion of the muscle by a trained nursing sister. For percutaneous vaccination a drop of vaccine was placed three-quarters of the way up the right arm in the deltoid region. The BCG vaccination tool was placed vertically above the drop of vaccine and the plunger pressed down for approximately 2 seconds. With the Biovac® tool, the plunger was released, the tool was rotated through 90° and the plunger was depressed again in order to make 18 needle holes.

Investigations

Peripheral blood mononuclear cell (PBMNC) and cord blood mononuclear cell (cord MNC) isolation. PBMNC and cord MNC were isolated by Ficoll–Hypaque (Histopaque-1077; Sigma Diagnostics, Inc., St Louis, MO) density gradient separation, cells were then washed in phosphate-buffered saline (PBS; Oxoid, Unipath Ltd, Hampshire, UK), and viable cells were adjusted to the optimal concentration for the specified tests. Cord blood and PBMNC reactivity from unvaccinated infants at 10 weeks served as a baseline response, for comparison to the vaccinated groups.

Lymphocyte proliferation and cytokine anlaysis. Lymphocyte proliferation was evaluated as previously described.[21] Briefly, 1×10^5 cells per well were plated in microculture and proliferation was measured in response to each of the following stimulants: $5 \cdot 75 \times 10^{-3}$ mitogenic units/ml phytohaemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, UK); tetanus toxoid (TT) antigen (formol toxoid – 10 Lf/0·5 ml; Lederle Laboratory, Wayne, NJ); purified protein derivative of *Mycobacterium tuberculosis* (PPD; Central Veterinary Laboratory, Weybridge, UK) at 3 μg/ml; *Mycobacterium tuberculosis* H37Rv strain (H37Rv) and *Mycobacterium bovis* BCG Japanese strain 172, each at a final concentration of approximately 10 organisms per macrophage [equivalent to 10^6 colony-forming units/ml (CFU/ml)]. Both of these organisms were heat killed. Duration of mitogen stimulation was 3 days and of antigen stimulation 6 days. Results were expressed as the difference in [^{3}H]thymidine incorporation (delta counts per minute) after subtraction of background unstimulated counts. Prior to addition of [^{3}H]thymidine, 100 μl of supernatant fluid was removed from each well and stored at −20° for cytokine evaluation. Commercially available antibody pairs were used for IFN-γ, interleukin-10 (IL-10) and IL-5 enzyme-linked immunosorbent assay (ELISA; PharMingen, San Diego, CA) following optimization. Recombinant cytokines were used as standards. Results are expressed in pg/ml after the deduction of

unstimulated samples. The lower limit of detection for the cytokines was 15 pg/ml.

CD4$^+$ CTL activity measured by adherent macrophage 16-hr sodium chromate ^{51}Cr-release assay. The cytotoxic capacity of effector cells was evaluated as previously described.[21] BCG-specific effector cells were generated by stimulating 1×10^6/ml PBMNC with heat-killed BCG Japanese strain (10^6 CFU/ml) in bulk culture for 6 days. Mycobacterial antigens H37Rv and BCG Japanese strain 172 (both at 5×10^5 CFU/ml and both heat killed) were added to monocyte-derived macrophage (MDM) target cells. TT was added as a control antigen. The effector to target ratios for each antigen were $10:1$, $3:1$ and $0.3:1$. The mean percentage killing was calculated as follows: % specific lysis = [(mean test c.p.m.)/(mean test c.p.m. + mean c.p.m. after Triton-X treatment of same wells)] × 100% − % spontaneous release; where c.p.m. are counts per minute. The % spontaneous release was calculated as follows: [(mean c.p.m. in spontaneous release wells)/(mean c.p.m. in spontaneous release wells + mean c.p.m. after Triton-X treatment of same wells)] × 100%. To obtain antigen-specific cytotoxicity, the percentage kill obtained in unpulsed targets was subtracted from percentage kill of mycobacterial antigen-pulsed targets.

Statistical analysis

Data were analysed using MICROSOFT EXCEL 97 and STATISTICA 5.5 (Statsoft, Tulsa, OK). Proliferation, cytotoxicity and cytokine results obtained in children post-vaccination were compared to those obtained pre-vaccination at 10 weeks and to cord blood results. Comparisons were also made between different groups to ascertain whether a statistical difference was obtained between strain of BCG, route of administration, age of vaccination and different percutaneous vaccination tools.

Non-parametric techniques were used as the underlying measurements could not be assumed to be normally distributed. Cytokine responses that were not detectable in the assays were assigned lowest rank in these tests.

As sample sizes are small, and multiple comparisons have been performed, all *P*-values should be interpreted as an indication of strengths of association between the variables.

The levels of significance shown in the figures result from application of the Mann–Whitney *U*-test in the comparison of unvaccinated and post-vaccinated infants.

Box and whisker plots indicate the minimum, maximum, median and interquartile range. Outliers are indicated by circles. In the median plots, medians are indicated as short lines. In Figs 4(a) 5(c) and 6(d) single outliers have been removed from the graphs in order to facilitate display, but they were included in all the statistical analyses.

RESULTS

In order to evaluate BCG immunogenicity in neonates, a comprehensive evaluation of cell-mediated immunity was performed. This comprised assessment of T-cell proliferation following *in vitro* mitogenic and mycobacterial antigenic stimulation, evaluation of antigen-specific cytotoxic capacity, and the balance of Th1-type (IFN-γ) and Th2-type (IL-4, IL-5 and IL-10) cytokines generated in response to BCG vaccination. Cord MNC and PBMNC reactivity in unvaccinated infants at 10 weeks served as a baseline response, for comparison to the vaccinated groups.

Proliferation responses

Responses to mycobacterial antigens, heat-killed BCG and H37Rv were measured in the five groups of infants. Responses to non-specific stimulation with the mitogen PHA, and the non-mycobacterial antigen TT served as controls (Fig. 1a–d). Similar PHA responses were observed indicating comparable T-cell reactivity in immune cells obtained from infants in all five vaccinated groups (Fig. 1a). These were significantly higher ($P < 0.001$) than pooled cord responses, which probably represents a maturational effect. Proliferative responses to heat-killed BCG and H37Rv (Fig. 1c,d) observed 10 weeks post-vaccination in the five groups of infants were significantly greater than baseline unvaccinated infants aged 10 weeks ($P < 0.003$). There was no statistical difference between the different infant groups. PPD responses showed a similar trend (data not shown). By contrast, proliferative responses to TT (Fig. 1b) were similar for baseline cords, unvaccinated infants, and post-vaccination responses in groups DID, JID, JPC Big and JPC Bio. Only the group who received Danish ID BCG delayed vaccination at 10 weeks of age (del DID) had significantly greater proliferative responses to TT, in comparison to the unvaccinated 10-week-old infant group ($P < 0.01$). This was to be expected as they had received more routine TT immunizations than the other infants.

Cytotoxicity

The results of antigen-specific cytotoxicity for all three effector to target (E : T) ratios are shown in Fig. 2. BCG vaccination induced higher levels of antigen-specific cytotoxicity against BCG and H37Rv pulsed autologous MDM in all five infant groups, as compared to control BCG-stimulated MNC cord effectors and the unvaccinated 10-week-old group. Highest antigen-specific kill was observed at a E : T ratio of $3:1$, as increasing CTL effectors resulted in higher levels of non-specific killing of unpulsed target cells. Figure 3 illustrates the non-specific or anomalous cytotoxicity observed with unpulsed and TT-pulsed MDM in the JPC Big infant group, this pattern was representative for all groups. Unexpectedly, BCG-stimulated effectors from the 10-week unvaccinated infants killed BCG-infected macrophage target cells (Fig. 2a); but this was less than in the vaccinated groups. Table 1 indicates the rank order of cytotoxic activity for the different groups. The table lists both the percentage of responders (defined as ≥ 5% antigen-specific kill) and the relative strength of CTL generated (percentage of the group with ≥ 10% antigen-specific kill) for all infant groups.
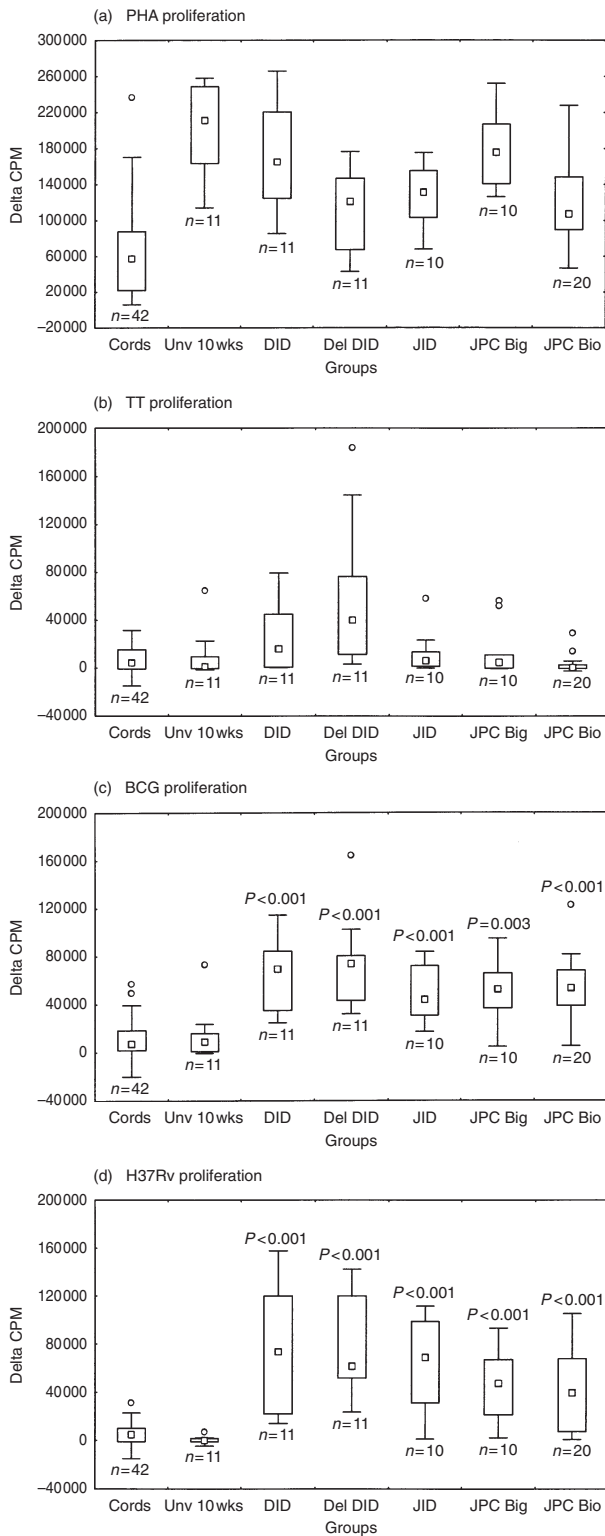
Figure 1. T-cell proliferative responses indicated as Δc.p.m. following stimulation with PHA (a), TT (b), BCG (c) and H37Rv (d). The five groups of neonates; Danish ID (DID), Danish ID administered at 10 weeks of age (del DID), Japanese ID (JID), Japanese PC via the Bignell® tool (JPC Big) and Japanese PC via the Biovac® tool (JPC Bio). The medians for the day 3 background
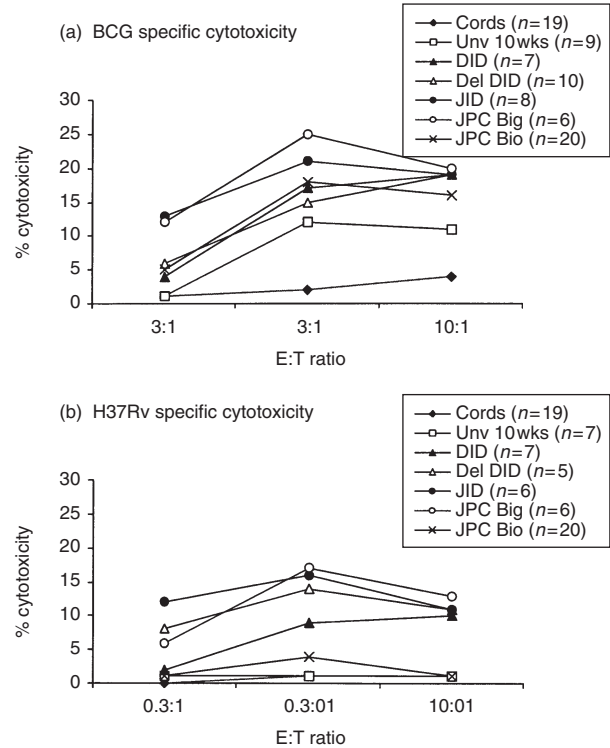

Figure 2. CTL activity of BCG-stimulated effectors against BCG-infected (a) and H37Rv-infected (b) targets is indicated as mean percentage specific cytotoxicity for three effector to target ratios 10:1, 3:1 and 0·3:1. Standard error of the mean was <5% for all groups. Note that values of the cord group in (b) are superimposed on the Unv 10 week group.
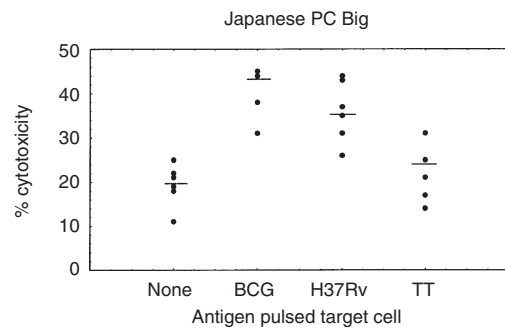

Figure 3. CTL activity of PBMNC obtained from the group of infants receiving Japanese PC via the Bignell® tool is indicated as percentage cytotoxicity at an effector to target ratio of 3:1. Medians are indicated as short lines.

unstimulated c.p.m. are as follows; cords, 4996; Unv 10 week, 862; DID, 1132; del DID, 565; JID, 479; JPC Big, 677; and JPC Bio, 5446. The medians for the day 6 background unstimulated c.p.m. are as follows; cords, 23135; Unv 10 week, 1799; DID, 2036;, del DID, 1034; JID, 896; JPC Big, 1533; and JPC Bio, 641.

Infants receiving JPC via the Bignell[®] tool showed the highest CTL activity on both these criteria, whilst the JPC Bio group had the lowest CTL activity. The other three vaccinated infant groups occupied an intermediate position between JPC Big and JPC Bio. It is particularly notable that JPC Bio infants had the lowest response rate of 55% against virulent H37Rv-infected targets, and the weakest level of cytotoxicity (30% of donors with ≥ 10% antigen-specific kill). According to these criteria, this group may be expected to be the most susceptible to pathogenic mycobacteria if CTL are an important correlate of protective immunity, as has been suggested.[15,16]

Cytokines

Th1-type and Th2-type cytokines were evaluated by measurement of IFN-γ, IL-5 and IL-10 secreted proteins in supernatant samples produced in response to stimulation by mycobacterial antigens (heat-killed BCG, H37Rv and PPD). In preliminary experiments IL-4 could not be measured in standard ELISA under conditions where IL-5 was readily detectable (data not shown), confirming the inability of even sensitive ELISA methodology to detect labile cytokines such as IL-4.[22] Cytokine results are shown in Figs 4, 5 and 6.

Response to BCG vaccination

For the mycobacterial antigens BCG and H37Rv, both IFN-γ and IL-10 production were significantly greater in all vaccinated groups as compared to the unvaccinated 10-week-old controls (Figs 4 and 5). Similar significant

Table 1. Percentage of infants with cytotoxic responses in vaccinated groups

	Responders* ≥ 5%	High Kill† ≥ 10%
BCG-specific cytotoxicity		
Cords (*n*=19)	32%	11%
Unv 10 week (*n*=9)	67%	56%
DID (*n*=7)	86%	86%
Del DID (*n*=10)	80%	80%
JID (*n*=8)	100%	100%
JPC Big (*n*=6)	100%	100%
JPC Bio (*n*=20)	80%	75%
H37Rv-specific cytotoxicity		
Cords (*n*=19)	21%	11%
Unv 10 week (*n*=7)	29%	14%
DID (*n*=7)	71%	57%
Del DID (*n*=6)	83%	83%
JID (*n*=6)	83%	67%
JPC Big (*n*=6)	100%	83%
JPC Bio (*n*=20)	55%	30%

*Responders are arbitrarily defined as ≥ 5% antigen-specific cytotoxicity.

†Strength of killing is indicated by percentage of infants ≥ 10% antigen-specific cytotoxicity.
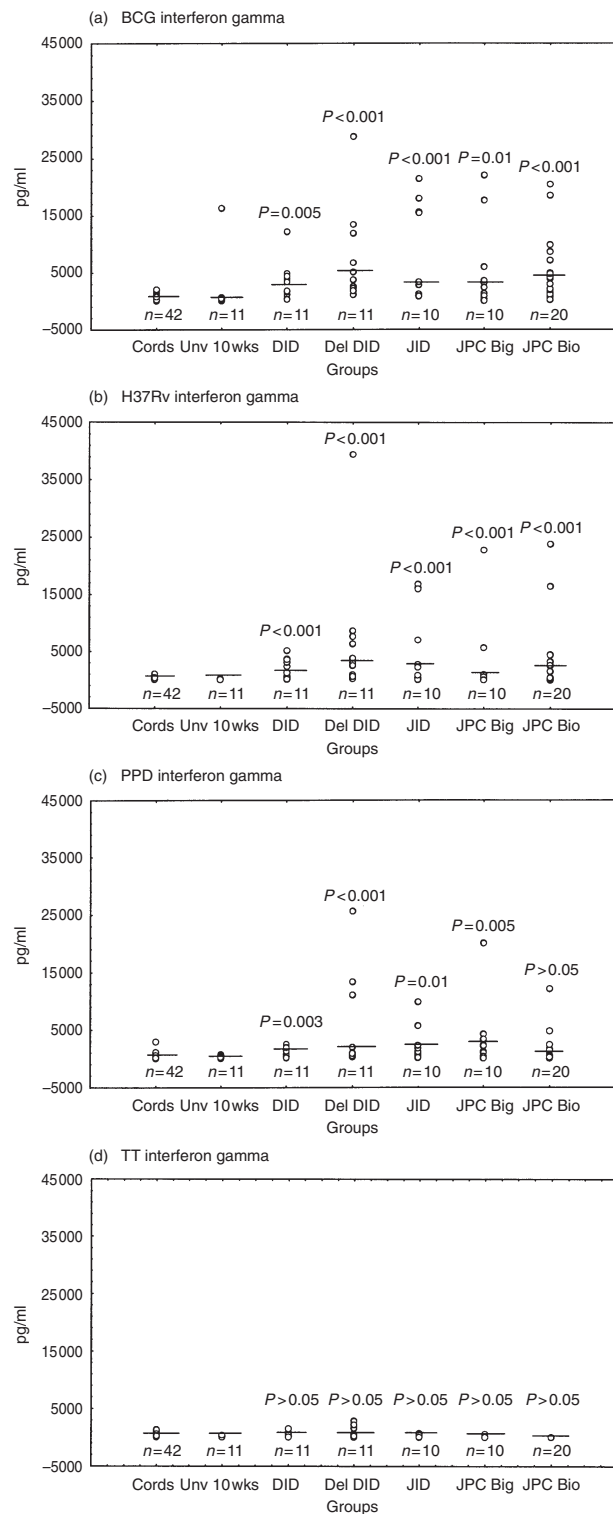
Data represents E : T ratio of 3 : 1.

Figure 4. Interferon-γ responses following stimulation with the mycobacterial antigens BCG (a), H37Rv (b), PPD (c) and TT (d) are indicated as pg/ml. Medians are indicated as short lines. A single outlier (61940 pg/ml) has been omitted from the del DID group in (a) to allow consistent scales and to facilitate comparison. The medians for the unstimulated samples are as follows; cords, 50 pg/ml; Unv 10 week, 82 pg/ml; DID, 37 pg/ml; del DID, 41 pg/ml; JID, 28 pg/ml; JPC Big, undetectable; and JPC Bio, undetectable.

differences were obtained with vaccinated as compared to cord responses for IFN-γ ($P < 0.001$). Between immunized groups there was no significant difference in IFN-γ and IL-10 production in response to mycobacterial antigens. There was a trend for higher responses in delayed vaccination (del DID) as compared to the same vaccine given at birth for IFN-γ ($P = 0.065$) and IL-10 ($P = 0.055$) production. With regard to mycobacteria antigenic stimulation of IL-5 production, significantly higher levels of BCG-induced IL-5 were obtained in infants receiving DID ($P = 0.016$) and JID ($P = 0.047$) than in the unvaccinated control group. PPD-induced IL-5 was significantly higher in infants that received BCG using the Biovac® tool than in the unvaccinated group ($P = 0.006$).

By comparison to mycobacterial antigen responses, the antigen TT elicited significantly greater IL-5 ($P < 0.001$) and IL-10 ($P = 0.013$) in the del DID group that had received more TT vaccination, as compared to the unvaccinated group (Figs 5d and 6d).

Cord blood responses

With regard to cord MNC reactivity, differential induction of Th-2-type cytokines preferentially occurred in response to BCG, as compared to H37Rv stimulation. As shown in Fig. 5, BCG and PPD both induced higher IL-10 than did H37Rv ($P < 0.001$). IL-10 was detectable in cord MNC supernatants of 41/42 donors stimulated by BCG and 40/42 with PPD. Higher levels were obtained for BCG (median 127, range 16–534 pg/ml) as compared to PPD (median 83, range 8–366 pg/ml). By contrast, a striking finding was that IL-10 was undetectable in 43% of cord MNC donors following H37Rv stimulation. In those donors that did respond, lower levels of IL-10 were obtained (median 19, range 2–81 pg/ml) in comparison to BCG and PPD stimulation. Production of IL-5 by cord MNC was higher in response to BCG than H37Rv or PPD stimulation ($P < 0.001$). The proportion of donors with undetectable IL-5 was 62% in response to stimulation by H37Rv, as compared to 57% for PPD and 10% for BCG.

There were no statistically significant differences in cord IFN-γ responses to the three mycobacterial antigenic preparations. However, H37Rv was the weakest antigen with undetectable IFN-γ in 57% of cord MNC responses, as compared to 31% undetectable for PPD and 19% undetectable for BCG.

Cord blood responses were thus biased towards the Th2-type cytokines IL-10 and IL-5 specifically in response to BCG, but not H37Rv. However, by 10 weeks, PBMNC from the BCG unvaccinated infants produced significantly less IL-10 than cord MNC in response to BCG ($P < 0.001$), H37Rv ($P < 0.003$) and PPD ($P = 0.025$). This may indicate a maturational response unique to IL-10 (rather than exposure to other vaccines or infections), since IL-5 and IFN-γ production did not significantly differ between cord and unvaccinated infants, in response to the same three mycobacterial antigenic preparations.
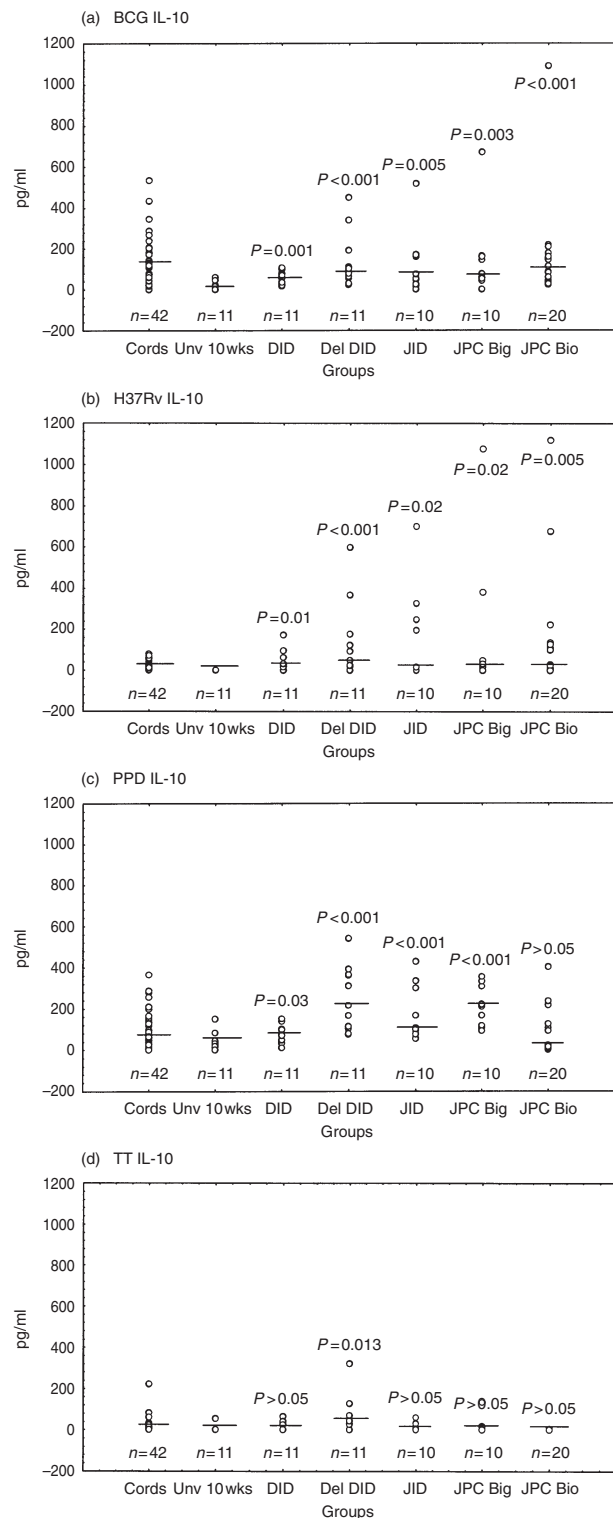
Figure 5. IL-10 responses following stimulation with the mycobacterial antigens BCG (a), H37Rv (b), PPD (c) and TT (d) are indicated as pg/ml. Medians are indicated as short lines. A single outlier (3661 pg/ml) has been omitted from the JPC Bio group in (c) to facilitate display. The medians for the background unstimulated samples are as follows; cords, 62 pg/ml; Unv 10 week, undetectable; DID, undetectable; del DID, undetectable; JID, 9 pg/ml; JPC Big, undetectable; and JPC Bio, 13 pg/ml.
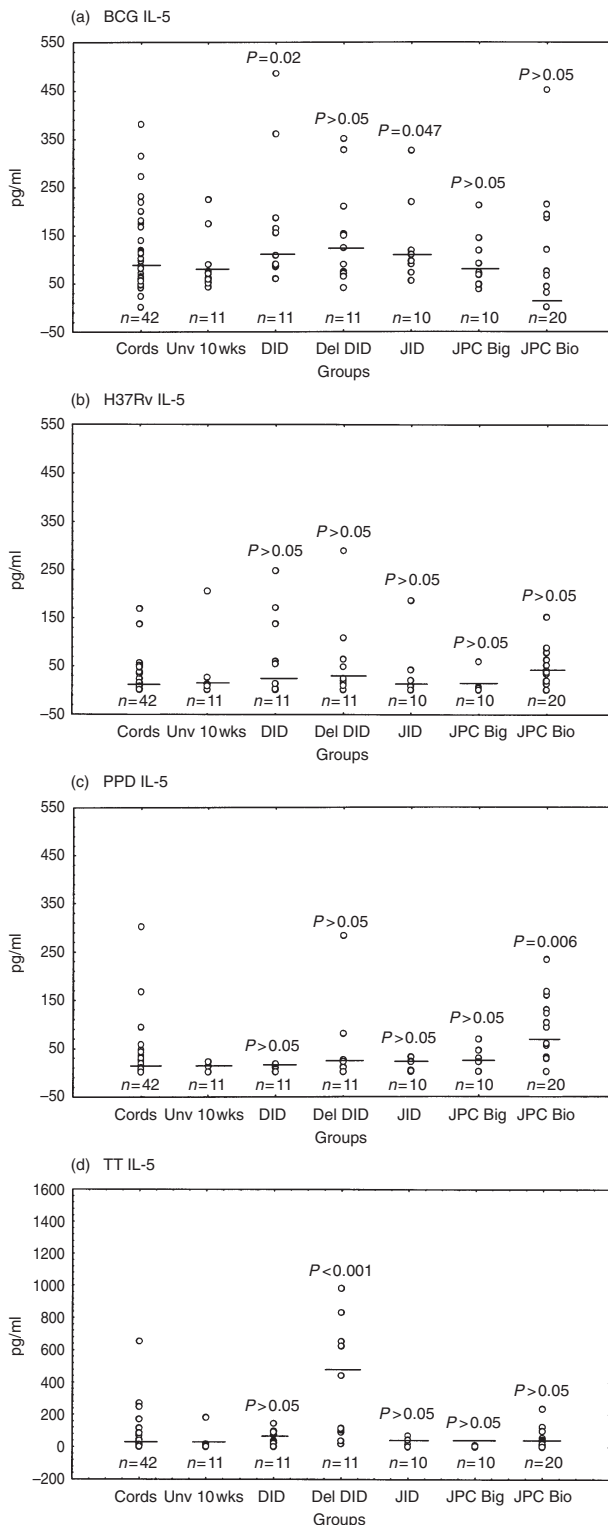
Figure 6. IL-5 responses following stimulation with the myco-bacterial antigens BCG (a), H37Rv (b), PPD (c) and TT (d) are indicated as pg/ml. Medians are indicated as short lines. A single outlier (13385 pg/ml) has been omitted from the del DID group in (d) to facilitate display. The medians for the background unstimulated samples are as follows; cords, 59 pg/ml; Unv 10 week, 67 pg/ml; DID, 83 pg/ml; del DID, 50 pg/ml; JID, 63 pg/ml; JPC Big, 80 pg/ml; JPC Bio, 60 pg/ml.

Th1-type versus Th2-type cytokine responses

Since Th1-type and Th2-type cytokines are cross-regulatory and mutually inhibitory, their net biological effect may best be assessed by comparison of cytokine ratios, rather than evaluation of individual cytokine levels. According to this hypothesis, BCG vaccination-induced protective immunity would be expected to correlate best with a significant augmentation in Th1/Th2-type cytokine ratio, over the cord and unvaccinated 10-week values (Table 2). The effect of delayed BCG vaccination on the immune response is evident from comparison of cytokine responses to BCG and H37Rv stimulation in the same group of infants. Table 2 compares cord MNC reactivity with that of PBMNC at 10 weeks (unv 10 week) and post-vaccination at 20 weeks of age (del DID). A striking finding was that the unvaccinated 10-week PBMNC IFN-γ : IL-10 ratio was significantly increased over the cord blood value selectively in response to BCG stimulation ($P = 0.0078$). This was due to both increased IFN-γ and reduced IL-10 production at this time-point. In comparing the effect of BCG vaccina-tion in the different infant groups at 10 weeks of age (Table 2), there was no significant increase in the IFN-γ : IL-10 ratio above the 10-week unvaccinated value, following BCG stimulation *in vitro*. This was due to the high unvaccinated 10-week value. IFN-γ : IL-10 ratios were significantly increased above the unvaccinated 10-week baseline values in response to H37Rv stimulation ($P < 0.02$). By contrast, a significant increase in IFN-γ : IL-5 ratio occurred in response to both BCG and H37Rv ($P < 0.001$) stimulation in all 10-week BCG-vaccinated groups as com-pared to unvaccinated 10-week baseline values ($P < 0.001$) (Table 2). It is also instructive to ask how variation in BCG vaccination at birth involving strain, route or tool specifically affects the increase in ratio of Th1-type : Th2-type cytokines (Table 2). Here, a significant increased IFN-γ : IL-5 ratio was observed only in response to BCG stimulation in comparison of the effect of tool (JPC Bio versus JPC Big) ($P < 0.01$) and age (del DID versus DID) ($P = 0.02$).

A correlation between a type 1 cytokine secretion profile (IFN-γ : IL-4 ratio) and cytotoxic activity has previously been noted at the clonal level.[23] We therefore evaluated whether this association held for bulk culture. Cord MNC and unvaccinated 10-week PBMNC had low median IFN-γ : IL-5 cytokine ratio values (<2) following BCG stimulation. As expected, this correlated with low cyto-toxicity against H37Rv- and BCG-infected target cells. As shown in Table 3, a positive relationship was noted between IFN-γ : IL-5 ratio and cytotoxic activity against BCG targets for all five BCG-vaccinated groups. A single exception occurs when the comparison is extended to cross-reactivity against H37Rv-infected targets, where the vaccination with the Biovac® tool resulted in dissociation between cytokine production and cytotoxic activity. This group exhibited the highest IFN-γ : IL-5 ratio (median 695) following BCG stimulation but minimal level of H37Rv antigen-specific kill (Table 3). Therefore, the general cor-relation between IFN-γ : IL-5 cytokine ratio and kill holds

Table 2. Comparison of Th1-type : Th2-type cytokine ratios in cord blood and infants vaccinated at birth or at 10 weeks

	IFN-γ : IL-10		IFN-γ : IL-5	
	BCG	H37Rv	BCG	H37Rv
Delayed vaccination group				
Cords	0·7 (0·0–3·2)	1·0 (1·0–1·0)	1·0 (0·0–2·3)	1·0 (0·1–1·0)
Unv 10 weeks	62·0 (1·2–366·0)	1·0 (1·0–95·0)	1·9 (0·5–7·1)	1·0 (0·1–20·0)
Del DID	61·8 (39·4–121·7)	51·3 (20·8–92·2)	33·6 (28·6–178·7)	133·3 (20·8–3817·0)
Route (JPC, JID), PC tool (JPC Big, JPC Bio), Strain (JID, DID) comparison				
JPC Big	39·1 (8·8–75·2)	58·4 (18·3–71·0)	45·0 (6·0–74·3)	288·3 (95·5–894·0)
JPC Bio	34·1 (22·6–78·0)	39·7 (23·8–116·6)	695·8 (36·3–4450·0)	40·0 (2·0–2915·0)
DID	49·0 (20·6–59·8)	96·0 (38·2–145·0)	18·3 (3·6–29·4)	93·5 (6·9–579·8)
JID	60·6 (31·5–105·0)	129·3 (48·9–343·0)	15·8 (13·2–47·3)	837·4 (58·9–7062·0)

Data expressed as median values, with 25th−75th interquartile range in brackets.

Table 3. Relationship between cytokine production and cytotoxicity

BCG strain	Variable	*In vitro* stimulation with BCG Japanese 172 IFN-γ : IL-5 ratio	BCG-specific kill percentage*	H37Rv-specific kill percentage*
JPC	TOOL-Big	45	25	17
JPC	TOOL-Bio	695	18	4
Del DID	Age	34	15	14
DID	Age/Strain	18	17	9
JID	Strain	16	21	16

*Antigen-specific kill for 3 : 1 E : T ratio.

in nine of 10 cases overall. However, no correlation was found between IFN-γ : IL-10 cytokine ratios and cytotoxicity.

DISCUSSION

Despite available control measures and routine BCG vaccination, tuberculosis remains epidemic in South Africa and many other developing countries. While several hypotheses have been proposed for variable protection following BCG vaccination,[4,24,25] generation of a suboptimum protective immune response is likely to be a contributory factor. Therefore, identification of any factors affecting the magnitude of immune response to BCG vaccination could potentially have an impact on vaccination practice.

Given the requirement for a Th1-type immune response in protective immunity against mycobacteria, we reasoned that vaccine efficacy would best be characterized by mycobacterial antigen-specific lymphoproliferative responses, IFN-γ production, high Th1-type/Th2-type cytokine ratios, and induction of CTL. These parameters were thus characterized in a group of infants from Cape Town, South Africa receiving BCG vaccine, with variation with regard to BCG strain, route, age and tool of administration. There were four main findings from this study. Comparable 10-week post-BCG vaccination *in vitro* cytokine and proliferative immune responses were found, with a trend for higher responses when BCG vaccination was delayed until 10 weeks and the use of Japanese strain via the intradermal route. Second, IL-10 levels in the supernatants from cord MNCs stimulated with BCG were higher than those in unvaccinated infants aged 10 weeks; a novel finding. Third, differential production of Th2-type cytokines IL-5 and IL-10 by cord MNC preferentially occurred in response to BCG (rather than H37Rv or PPD) stimulation and finally, we found evidence that BCG can prime for the generation of neonatal CTL but that the specificity of CTL activity was strongly influenced by the tool utilized in the vaccination procedure.

The ratio of IFN-γ : IL-10 cytokines produced by CD4$^+$ Th1 clones has been found to be of critical importance in determining their pro- or anti-inflammatory functional bias.[26] The ratio of Th1-type to Th2-type cytokines produced by PBMNC *ex vivo* has also indicated significant systemic bias in cytokine production towards either Th1 or Th2 dominance in autoimmune disease.[27] The IFN-γ : IL-10 ratio has proved useful in documenting diurnal variation in the balance of Th1- and Th2-type cytokines.[28] These considerations are also important in clinical tuberculosis where a Th1-type cytokine response is considered essential for protective immunity,[6] while significant Th2-type cytokine production correlated with adverse clinical outcome.[29] In animal experiments, there was a strong correlation with vaccine protective efficacy

and Th1/Th2 balance.[30] We therefore compared the ratios of Th1-type to Th2-type cytokines following BCG vaccination. A striking finding was high IL-10 production by cord MNC preferentially in response to BCG stimulation. BCG-induced IL-10 secretion had diminished by 10 weeks of age. This was associated with an increased IFN-γ: IL-10 ratio that may be the result of maturation of the immune response or may represent the effect of other vaccines received by neonates in the first 10 weeks of life. However, this was a selective effect and was not accompanied by significant changes in IFN-γ: IL-5 ratio in the unvaccinated 10-week-old group.

Considerable data exist on cord blood response to mitogen and allogeneic cell stimulation.[31,32] Cord blood displays an altered cytokine profile in response to mitogen stimulation with defective IFN-γ and IL-10 responses; this progressively increases in the first year of life.[31] Developmental delay in neonatal IL-10 production has been attributed to decreased expression of tumour necrosis factor-α and its receptors.[33] However, IL-10 production in response to BCG stimulation by human cord MNC has not been well studied, although murine studies have previously documented a neonatal Th2 bias.[7–9] The current study made use of heat-killed BCG and H37Rv antigens that probably enhanced the expression of heat-shock proteins, these may have represented a 'danger' signal to Th2 anti-inflammatory cells.[34] It is possible that heat-shock protein or related antigens may be more immunogenic in BCG than H37Rv to account for our findings. Whilst BCG and H37Rv were grown in identical media and CFUs were enumerated, we have no data on viability of the preparations prior to heat killing. It cannot therefore be excluded that the preheat-treated BCG may have contained greater numbers of dead organisms as a trivial explanation for induction of higher Th2-type cytokine levels. However, this explanation would not account for the reduction in BCG-stimulated IL-10 by 10 weeks. Finally, helminth infection may have been a factor in our study population.[35,36] Cord blood T cells have been shown to be hyporesponsive to IL-12 but respond to allergens and helminth infections with production predominantly of the Th2-type cytokines IL-4, IL-10, IL-13.[37]

The implication of IL-10 production in response to BCG stimulation for host protective immunity remains to be determined. IL-10 is conventionally considered an immunosuppressive, Th2-type cytokine in view of its inhibition of macrophage pro-inflammatory cytokine production and Th1-type responses.[38] In keeping with this, IL-10 has been shown to antagonize macrophage function in mycobacterial infection[39] and IL-10-deficient mice exhibit increased antimycobacterial immunity.[40] It is also of interest that prenatal sensitization to helminths has been shown to be associated with decreased Th1-type immunity induced by BCG vaccination,[41] although IL-10 was not measured in that study. On the other hand, there is also evidence that IL-10 plays an important role in protective immunity in several infections including *Candida*, trypanosomiasis and tuberculosis. Thus, IL-10 has been shown to be required for optimal development of CD4$^+$ Th1 cells in

mice infected with *Candida albicans*.[42] Recent experimental evidence indicates an important role for IL-10 in survival and resistance of mice infected with African trypanosomes.[43] CD4$^+$ T-cell clones isolated from bronchoalveolar lavage of patients with active pulmonary tuberculosis produced both IFN-γ and IL-10.[44] While IL-10 production may represent an attempt to limit local tissue damage, a role for IL-10 in protective immunity cannot be excluded. Thus IL-10 treatment *in vitro* endows human dendritic cells with the ability to inhibit intracellular growth of virulent mycobacteria.[45] Reduced stimulation of IL-10 production by virulent H37Rv in the unvaccinated neonate could thus favour intracellular survival of the organism within parasitized dendritic cells. Further work is needed to establish the biological outcome of opposing IL-10 activity in mycobacterial infection, as in the case of *Aspergillus* infection.[46]

While CTL have been considered an important component of protective immunity against mycobacteria,[15,16] previous studies have been limited to adults.[19,47,48] To the best of our knowledge, this is the first study to measure cytolytic activity in neonates. Our hypotheses were that failure to prime for CTL activity could be a factor contributing to inadequate protective immunity following BCG vaccination, and that the ability of BCG to prime for CTL activity against H37Rv would represent the best measure of protective cytolytic capacity. In this context, it is interesting to note that the lowest cytolytic activity was observed against H37Rv-infected macrophages in neonates vaccinated with the Biovac® tool. Importantly, this represented a selective effect on CTL activity and did not affect cytokine production, nor result in any skewing of Th1: Th2 ratio (Table 2). Indeed, the Biovac® tool gave the highest IFN-γ: IL-5 ratio following BCG stimulation (Table 2). Aside from this exception, a correlation was found between increased IFN-γ: IL-5 ratio and cytotoxic activity. Increased IFN-γ: IL-5 ratio was also observed for an age-related maturational vaccine response (higher with both BCG and H37Rv in del DID than DID). These data suggest that IFN-γ: IL-5 ratio may provide a useful general indicator of the Th1-type response and vaccine efficacy. These findings, including an opposite trend in CTL activity and cytokine production dependent on the vaccine tool employed, may have important implications for correlates of protective immunity in mycobacterial vaccine studies. Previously, adjuvant-induced IFN-γ levels did not clearly correlate with protective immunity,[30] suggesting the need for additional correlates of protection. The non-specific or anomalous killing described here has also previously been reported[19,47,48] and may be due to 65 000 MW heat-shock protein.[49] The relevance of generation of CTL activity following BCG vaccination for protective immunity will need to be addressed by additional prospective studies. These will also need to evaluate the relative contribution of distinct CTL subsets ($\gamma\delta^+$, CD4$^+$, CD8$^+$) and cytolytic mediator molecules such as perforin, granzymes and granulysin.[14,18]

Tuberculosis remains widespread in many developing countries 5 years after the World Health Organization

declared it a global emergency, despite a concerted effort to combat the disease. The best hope of stemming the epidemic lies in the development of a more effective vaccine, since prospects for new drug development are limited by cost, while their efficacy would be limited by increasing multidrug resistance. Current efforts are directed at improved definition of immunological correlates of protective immunity,[14,50] improved DNA-based modes of vaccination[51] and molecular analysis of the mycobacterial genome.[52,53] However, successful implementation of novel vaccine candidates in the future will need to take several additional factors into account. These include the impact of variation in vaccination strategy on immune parameters as documented in this study, host population genetic factors,[54,55] and environmental influences including parasitic infections.[36,37,41] We noted considerable donor variation in the immune response to BCG vaccination and larger studies will be required to confirm the trends reported here and to develop improved BCG vaccination strategies.

ACKNOWLEDGMENTS

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